Extracellular Binding Sites of IGA Anti-Jejunal Antibodies on Normal Small Bowel Detected by Indirect Immunoelectronmicroscopy

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Patients with dermatitis herpetiformis (DH) have IgA deposition in the papillary dermis and in the lamina propria of the small bowel. In addition, most of DH patients' sera contain IgA class anti-reticulin antibodies, anti-endomysium antibodies (EMA), and anti-jejunal antibodies (JAB) during times of gluten intake. In previous studies, JAB and EMA seemed to be identical and related to the group of anti-reticulin antibodies.

In the present study, pre-embedding en bloc immunoelectronmicroscopic methods were applied for analysis of the ultrastructural binding sites of JAB on monkey and rabbit small bowels. These substrates were incubated with sera from DH patients strongly positive for JAB. Simultaneous investigations with the PAP technique and with 5 nm gold-labeled protein A or second antibodies visualized the bound IgA identically: it was associated with collagen fibrils underlying the epithelial and cryptal basement membranes and with collagen fibrils around capillaries. Staining was also detected along the endomysial collagen fibrils of smooth muscle layers, around elastica and smooth muscle cells of blood vessel walls, and along collagen fibrils near smooth muscle cells in the lamina propria. Neither the peroxidase product nor gold deposition was detected directly on the fibers, but was associated with amorphous material surrounding collagen fibers of different diameters. The distribution of JAB-stained structures corresponded to the localization of reticulin network of the small bowel.

Our data indicate that JAB recognize an antigen or antigens associated with an amorphous component of the reticulin-collagen structure of jejunum and may have identical binding sites, as anti-reticulin antibodies and EMA. *J Invest Dermatol 96:228–233, 1991*

Dermatitis herpetiformis (DH) is a pruritic blistering disease of the skin with a characteristic immunohistologic marker, a unique granular deposition of IgA in the tips of dermal papillae. The many immunologic findings in DH have given rise to the view that it is an immunologic disorder [1].

Most patients with DH suffer from a gluten-sensitive enteropathy (GSE), which is characterized by villous atrophy of the small bowel mucosa. This atrophy is caused by gluten intake, but is usually less severe than that of celiac disease (CD). Although circulating IgA antibodies against skin structures have not been found, the sera of most DH patients contain antibodies that are also characteristic of CD patients, including IgA anti-reticulin antibodies [2]. "Reticulin or reticular fibers," or "reticulin," show distinctive staining characteristics: they are strongly PAS (periodic acid Schiff) positive and argyrophilic, meaning they can be detected by silver impregnation (Gömöri staining) [3]. Sera of most untreated DH patients also contain IgA anti-endomysium antibodies (EMA) and anti-jejunal (JAB) antibodies [4,5], which can be detected on monkey esophagus and human small bowel, respectively. JAB and EMA were found to be very similar in immunofluorescence (IF) studies [7] and were related to the anti-reticulin antibodies. JAB were present in about 70% of untreated DH patients, disappeared with gluten-free diets (GFD), and reappeared after gluten challenge [5,6]. Even though the presence of JAB and EMA depend upon gluten (gliadin) intake, they are different from anti-gliadin antibodies [7].

Recently, by direct IF, specific IgA staining was seen in jejunal

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Abbreviations:

BM: basement membrane
BSA: bovine serum albumin
CD: celiac disease
DAB: diaminobenzidine
DH: dermatitis herpetiformis
EM: electronmicroscopy
EMA: anti-endomysium antibodies
FITC: fluorescein isothiocyanate
GFD: gluten-free diet
GSE: gluten-sensitive enteropathy
IEM: immunoelectronmicroscopy
IF: immunofluorescence
JAB: anti-jejunal antibodies
PAP: peroxidase-antiperoxidase
PAS: periodic acid Schiff
PB: phosphate buffer
PBS: phosphate-buffered saline

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biopsy samples of 12 DH and two CD patients taken during times of gluten ingestion [8]. This unique IgA deposition in the diseased jejunum correlated with the presence of serum JAB. The binding of circulating JAB on normal human jejunum and the deposition of IgA in the biopsy samples of diseased jejunum gave similar staining patterns in IF studies.

In this work, immunoelectronmicroscopy (IEM) was applied for analysis of the binding sites of JAB within monkey and rabbit jejunum. Although IEM studies have investigated the distribution of IgA in the skin and oral mucosa of DH patients [9–14], the binding sites of circulating IgA antibodies, such as EMA, JAB, or antireticulin antibodies, have not been previously studied.

MATERIALS AND METHODS

Sera of DH and Control Patients For indirect IF and IEM, sera of two untreated children with DH, characterized by the typical rash and granular IgA deposition in the dermal papillae, were used. Both had severe jejunal atrophy on small bowel biopsy and JAB in the blood. JAB-negative sera from two children with bowel disease but without CD and DH, who had no villous atrophy on intestinal biopsies, were used as negative control sera. In addition, the serum of a normal child without JAB was also used as a control.

Sera were stored at −35°C until used for the present study.

Gömöri Staining Silver staining was carried out on cryostat sections of substrate tissues as described previously [3].

IF Studies The upper parts of the small bowels of a white house rabbit and a monkey were removed, washed in ice-cold PBS (0.01 M, pH 7.4) for 20 min, embedded in OCT (Lab Tek Ames Co, IN), quick frozen in liquid nitrogen, and stored at −70°C until sectioned. Indirect IF examination for JAB was carried out on 4-μm cryostat sections with DH and control sera and with fluorescein isothiocyanate (FITC)-labeled goat anti-human IgA (F/P molar ratio 2.8, diluted 1:40) (ATAB, FRG) as the second antibody [5].

Preembedding Indirect En Bloc Immunoelectronmicroscopy (IEM)

Gold Labeling for Detection of JAB on Unfixed Monkey Tissue: The method described by Sakai et al [15] was modified as follows: small pieces of the small bowel were washed in ice-cold 0.01 M PBS (pH 7.4) containing 1% bovine serum albumin (BSA) and 20 mM NaN_3), sliced into 0.5–1-mm–thick sections and incubated overnight at 4°C with DH or control sera diluted in 1:20 in PBS. After washing in PBS for 2 h, tissue samples were incubated for 2 h with rabbit anti-human IgA (DAKO, Glostrup, Belgium) diluted 1:15 in PBS. After washing in PBS for 2 h, tissue samples were exposed for 2 h to a 5-nm protein-A gold complex (Janssen Life Science Products, Belgium), diluted 1:10 in 1% BSA-TRIS-HCl buffer (TRIS 20 mM, NaN_3 20 mM, pH 7.6). After washing, the tissue was postfixed in Karnovsky [16] and 1% OsO_4 solution and was embedded in Epon 812 (SERVA, FRG).

Peroxidase-Antiperoxidase (PAP) Labeling for Detection of JAB on Unfixed Monkey Tissue: All steps were done as described above, except that the sections were incubated with rabbit antihuman IgA (DAKO, Denmark), diluted 1:15, for 2 h, with swine anti-rabbit immunoglobulins (DAKO), diluted 1:15, for 2 h with PAP-rabbit complex (DAKO), diluted 1:20, for 1 h, and with diaminobenzidine (DAB) (SERVA), as described previously [17].

Gold Labeling for Detection of JAB on Prefixed Rabbit Tissue: The method by Horvath and Palkovits [18] was adapted as follows: small pieces of jejunum were washed in PB (0.1 M, pH 7.4), fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in PB for 2 h at room temperature, washed, cut into 0.5–1-mm–thick strips, rinsed in a graded series of 10, 20, and 30% sucrose in normal saline (0.9%), and quick frozen in liquid nitrogen. The samples were then incubated at 4°C for 48 h with patient or control sera diluted 1:20 in PBS (0.01 M, pH 7.4). After washing in PBS, the tissue strips were incubated with a mouse monoclonal antibody to human IgA (Zymed Laboratories, USA) diluted 1:10 in PBS, and then with 5-nm gold-conjugated goat anti-mouse IgG (Janssen Life Sciences, Belgium), diluted 1:15 in TRIS-HCl buffer (see above) at room temperature. Incubation and washing times for all of these steps were 2 h. The samples were postfixed in Karnovsky solution and 1% OsO_4 and then embedded in Epon, as described above.

For electronmicroscopy (EM), 60–90-nm sections were cut on an Ultracut ultramicrotome (Reichert Scientific Instruments) and mounted on uncoated and formvar-coated copper grids. Grids were stained with 2% uranyl-acetate for 30 min and with Reynold's lead citrate [19] for 8 min and examined on a Philips transmission electronmicroscope (model 300), operated at 80 kV.

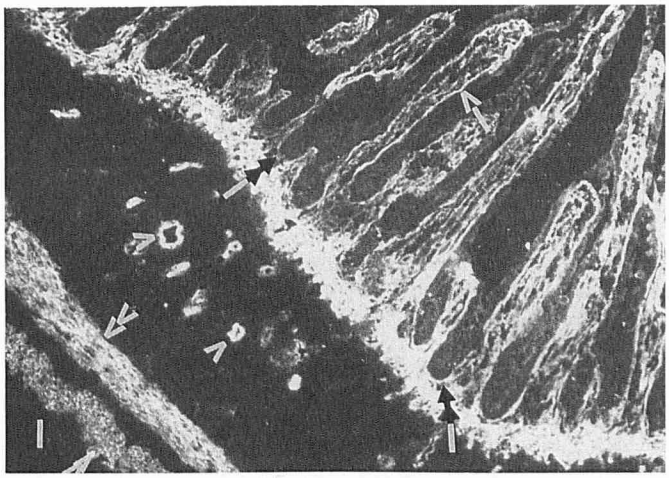

Figure 1. Indirect IF detection of binding of IgA from a DH serum to rabbit jejunum. The binding appears as tubular fluorescence underlying the villous *(arrow)* and cryptal *(arrow with arrowhead)* BM, as a network-like staining in the lamina propria, as staining along the muscularis mucosae, as staining around blood vessels of the submucosa *(arrowhead)*, and as staining along the endomysium of the smooth muscle layers *(double arrowheads)* (magnification ×114).

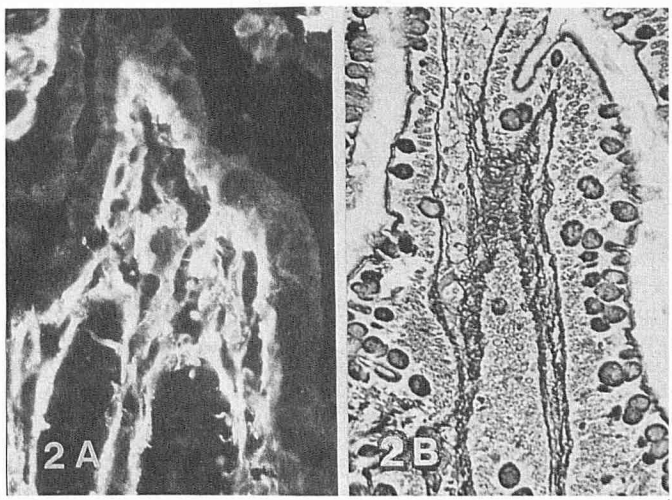

Figure 2. *A*, Indirect IF detection of binding of IgA from a DH serum in a tubular or band-like pattern underlying the villous BM of rabbit jejunum (magnification ×266); *B*, Gömöri silver staining of the villi of rabbit jejunum (magnification ×285).

RESULTS

Indirect IF Studies Incubation of cryostat sections of monkey and rabbit jejunal tissue with DH sera resulted in detection of JAB in identical locations on the two substrates [5] (Fig 1). JAB binding was detected as a band-like or "pearl-like" fluorescence underlying the epithelial and cryptal basement membranes (BM), and as a halo-like, interdigitating fluorescence in the cryptal lamina propria (Fig 2*A*). In addition, fibrillary staining was also noted within and around the blood vessel walls of the submucosa, as well as along the sarcolemma of the smooth muscle layer (Fig 1, 2*A*). The binding of the IgA JAB from the two DH sera was identical on both substrates, and the JAB titers ranged between 1:320–1:640. With control sera, no JAB were detected. The sites of JAB binding on jejunum were similar to those of argyrophilic fibers identified by Gömöri staining (Fig 2*B*).

Pre-Embedding Indirect Immunoelectronmicroscopic Studies Results obtained on unfixed and prefixed jejunum with gold-labeled protein-A or second antibodies and with the PAP technique were similar. However, gold labeling yielded a more detailed morphology of the labeled elements. Gold particles and the peroxidase reaction product, marking the binding sites of the IgA, were found to be located near collagen fibers and fibrils of different diameters. The sites of deposition were associated with grains of amorphous material surrounding the collagen fibers at regular intervals (Fig 3). In addition, gold and DAB deposition along the very fine fibrillary structures between collagen fibers was also observed (Fig 3). When several sections taken from different sites of the jejunum were compared, deposition of gold particles and the peroxidase product, indicating JAB binding, showed the following distribution: (a) along collagen fibrils underlying the epithelial and cryptal BM (Fig 4*A*); (b) along collagen fibrils around the capillaries underlying the epithelial and cryptal BM, as well as around lymphatics of lamina propria (Fig 4*C,D*); (c) adjacent to collagen fibrils around smooth muscle cells and fibroblasts of the villous and cryptal lamina propria; (d) along endomysial collagen fibrils of the smooth muscle layer (Fig 4*B*); and (e) along collagen fibrils between the lamina elastica and the smooth muscle cells of blood vessels.

Control Experiments In experiments that employed JAB-negative human sera and the PAP technique, we only saw the product intracellularly within endothelial cells, monocytes, and fibroblasts, but not at the extracellular locations seen with DH sera. Similar control experiments with gold-conjugated protein-A or second antibodies were negative or showed faint, irregularly scattered binding of gold particles (Fig 5*A,B*).

The results of the present study, obtained by three different ultrastructural methods, were uniform and clearly demonstrate the extracellular binding sites of IgA JAB.

DISCUSSION

The present study demonstrates the ultrastructural binding sites of IgA JAB from DH sera. To diminish the possible errors from nonspecific staining along collagen fibers, we simultaneously applied three different indirect IEM techniques, along with the appropriate controls.

JAB could be detected along collagen fibrils underlying the epithelial and cryptal BM, along pericapillary fibrils in the villous and cryptal lamina propria, and along collagen fibrils adjacent to smooth muscle cells and fibroblasts. Binding of IgA JAB along collagen fibrils was also detected in blood vessel walls and in the endomysium of the smooth muscle layer.

The binding sites of JAB by IF were found to be similar to the distribution of the jejunal "reticulin" fibers, which stain strongly with a silver stain (Fig 2). The reticular network of intestinal mucosa consists of fine nets of collagen fibers within the lamina propria supported by a spongy framework of interconnecting flattened fibroblasts. This network surrounds the epithelial and cryptal basal lamina, as well as the underlying capillaries [20].

The argyrophilia of reticulin, as well as the strong PAS reactivity, are thought to be due to the high carbohydrate content of the fibers or to the proteoglycan content of the interfibrillar matrix [21,22].

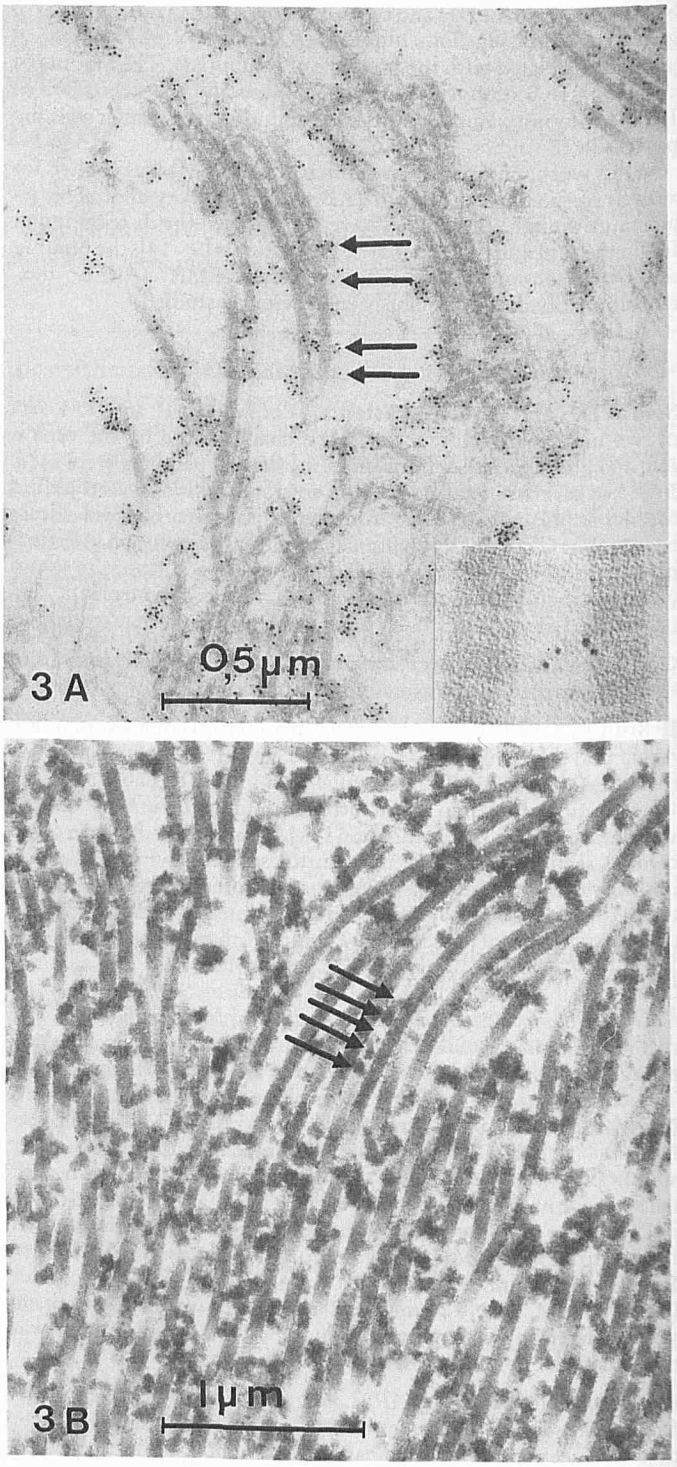

Figure 3. *A*, Indirect IEM deposition of gold particles, marking the sites of IgA binding along a collagenous structure of the villous lamina propria of the unfixed monkey jejunum (magnification ×43,700); *B*, peroxidase product, detected by indirect IEM, marking the binding of IgA from a DH serum along collagen (reticulin?) fibers of the unfixed monkey jejunum (magnification ×26,410).

Pras and Glynn [23] isolated an argyrophilic non-collagenous protein from the reticulin tissue of parenchymal and lymphatic organs. The argyrophilia of granulation tissue, which has reticulin different from that in parenchymal tissues, is probably due to proteoglycans [24].

Collagen type III and fibronectin form loose networks in paren-

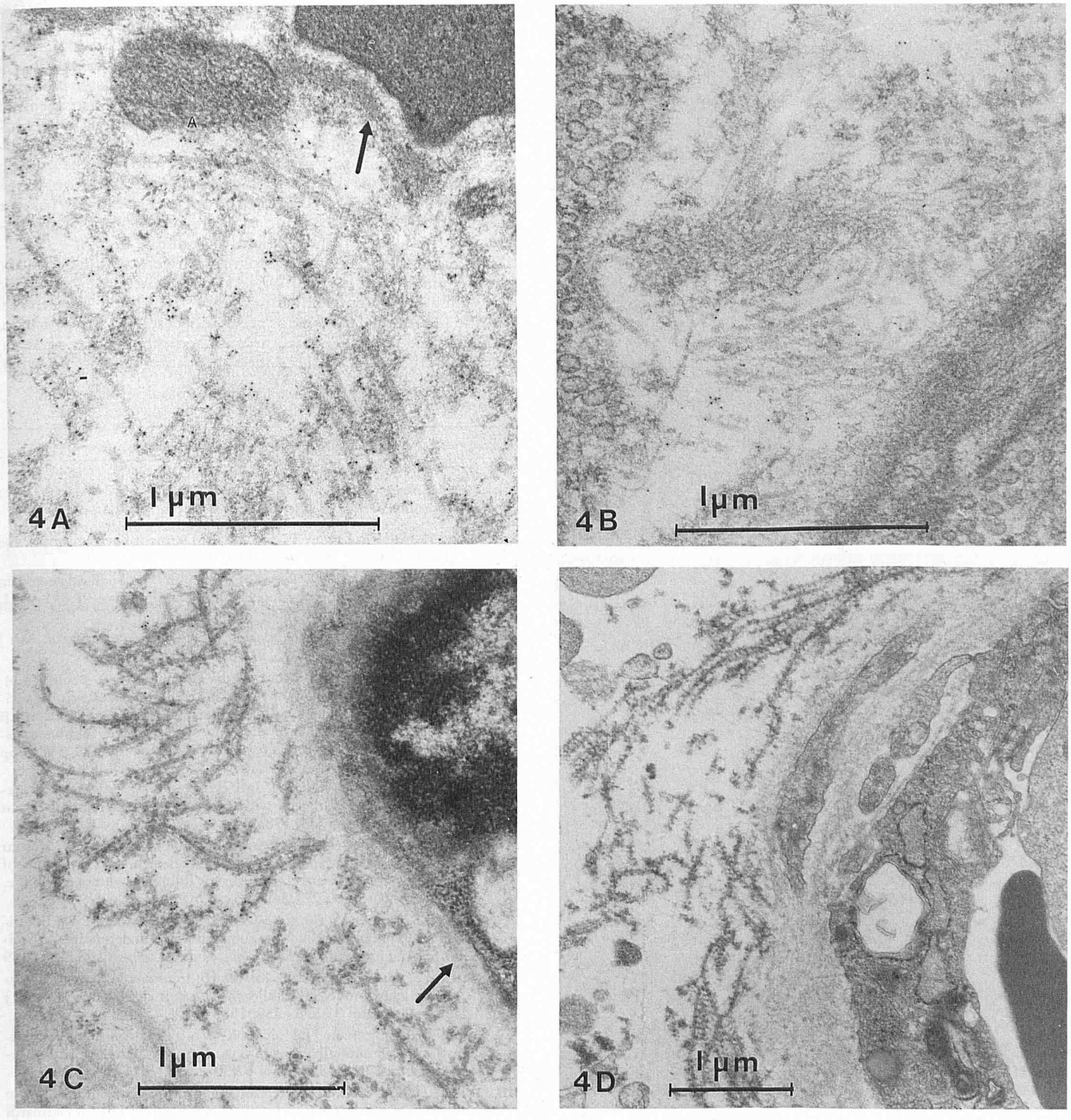

Figure 4. *A*, Gold-labeled antibody deposition, marking the binding of IgA from a DH serum, along collagen fibrils underlying the epithelial BM *(arrow)* of prefixed rabbit jejunum (magnification ×43,605); *B*, gold particles, marking the binding of IgA from a DH serum, along endomysial collagen fibrils between smooth muscle cells of fixed rabbit jejunum (magnification ×43,700); *C*, gold particle deposition, marking the binding of IgA from a DH serum, along pericapillary collagen fibrils of unfixed monkey jejunum (*arrow* to BM) (magnification ×35,720); *D*, peroxidase product, marking binding of IgA from a DH serum, along pericapillary collagen fibrils of unfixed monkey jejunum (magnification ×21,185).

chymal organs and blood vessels and are distributed similarly to reticulin fibers [25–27]. Because of the co-localization and the presence of copolymers of collagen I and III within the same fibers [28–31], it is impossible to distinguish them ultrastructurally.

"Reticulin" may, in fact, correspond to a group of different fibrillar and amorphous substances: collagen I fibers, collagen III fibers, fibronectin, or one or more non-collagenous proteins and proteoglycans [25,26,32]. Antibodies to reticulin may react with distinct conformations of these individual components or with distinct antigenic sites located on one or more of these components. The present study demonstrates that JAB recognize an antigen or antigens within an amorphous component associated with collagen (reticulin?) fibers of different diameters.

Antibodies against a 90-kD reticulin glycoprotein, isolated by

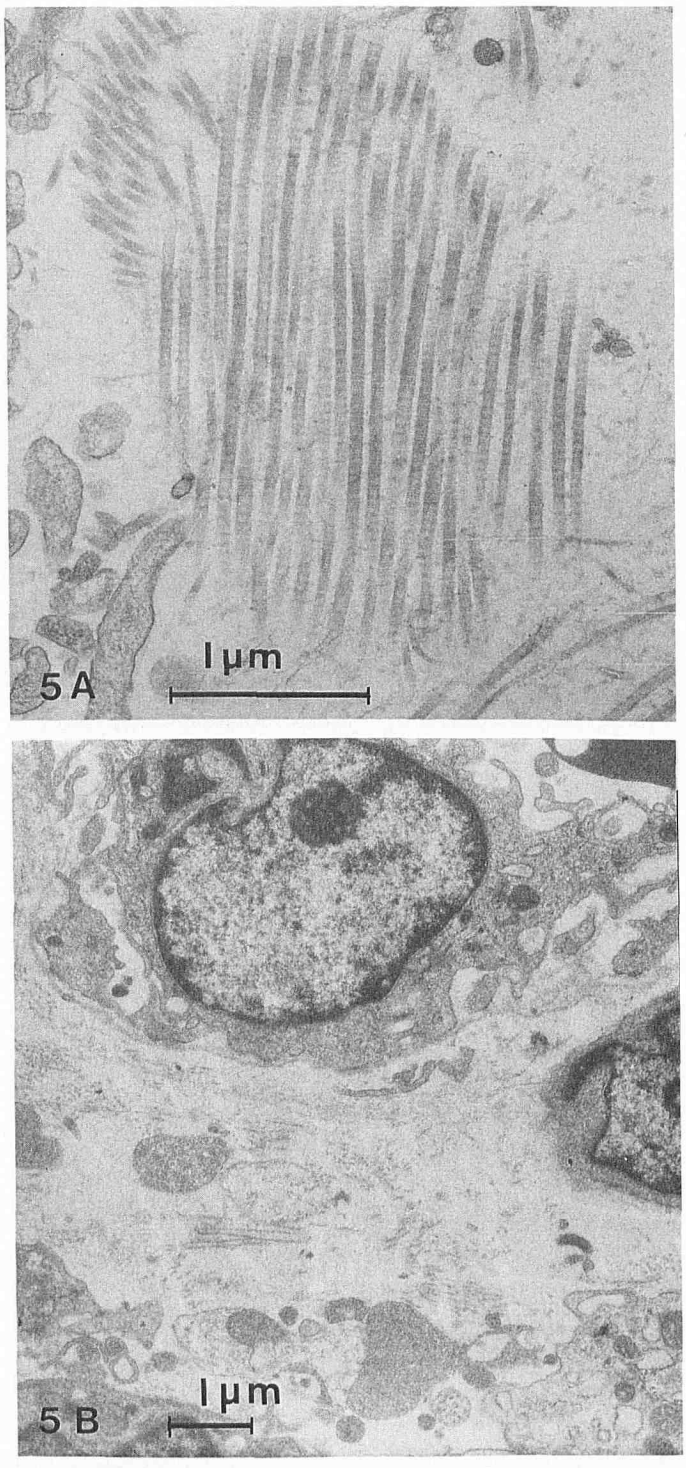

Figure 5. *A*, Absence of gold particles on unfixed monkey jejunum after indirect IEM with a control serum (magnification ×26,410); *B*, no peroxidase product along pericapillary collagen fibers on unfixed monkey jejunum after indirect IEM with a control serum (magnification ×10,735).

Maury and Teppo [33], can also be detected in DH and CD, but seem to be different from anti-reticulin antibodies [34].

In contrast to other serologic markers in DH and CD, JAB were shown to be directed against the target organ of the gluten sensitivity, the small bowel. The pattern of IgA deposition within the lamina propria of CD and DH patients with active GSE is very similar to the binding pattern of JAB on normal jejunum [8]. The binding sites of JAB may correspond to the sites of the first ultrastructural changes in the villous connective tissue detected after gluten challenge in patients with GSE [35]. Simultaneous IF studies revealed the reappearance of IgA deposition at this site [36].

In DH patients, the presence of JAB correlated with small bowel disease but not with the activity of skin disease [5]. JAB are probably induced in association with the immunologically mediated jejunal damage caused by ingestion of wheat protein by patients with gluten sensitivity. Most of these patients have bowel disease only without skin disease (CD patients). Only patients who have the specific IgA deposition in the skin have the associated skin disease we recognize as DH. Binding of JAB or EMA to the papillary dermis has not been detected. These facts point to the involvement of factors besides IgA JAB in the pathogenesis of skin disease in DH.

Our data indicate that JAB recognize antigens within an amorphous component of the small bowel associated with collagen fibers and with a distribution similar to that of "reticulin". We believe that the JAB may be the same as previously described anti-reticulin antibodies and EMA. Whether these antibodies might play a role in amplifying the gluten-induced intestinal damage in patients with DH is unknown.

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